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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/18982 <b>(22) International Filing Date:</b> 18 August 1999 (18.08.99) <b>(30) Priority Data:</b> 60/097,120 19 August 1998 (19.08.98) US 09/376,911 18 August 1999 (18.08.99) US <b>(71) Applicant:</b> NORTH AMERICAN VACCINE, INC. [US/US]; 10150 Old Columbia Road, Columbia, MD 21046 (US). <b>(72) Inventors:</b> MICHON, Francis; 4401 Rosedale Avenue, Bethesda, MD 20814 (US). HUANG, Chun-Hsien; 9815 Bald Cypress Drive, Bethesda, MD 20850 (US). UITZ, Catherine; 4126 North 34th Road, Arlington, VA 22207 (US). <b>(74) Agents:</b> SONNENFELD, Kenneth, H. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 22 June 2000 (22.06.00)
<b>(54) Title:</b> IMMUNOGENIC $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE PROTEIN CONJUGATE USEFUL AS A VACCINE PRODUCED USING AN N-ACRYLOYLATED POLYSACCHARIDE  <div style="text-align: center;"> <p> <math>\text{PS-NHAc (1)} \xrightarrow[\text{Enzyme}]{\text{Base}} \text{PS-NH}_2 \text{ (2)} \xrightarrow{\text{CH}_2=\text{CHCOCl}} \text{PS-NH}_2\text{C(=O)CH=CH}_2 \text{ (3)}</math>  <math>\text{(3) + eNH}_2\text{-Lys-Pro} \xrightarrow{\text{pH 9-10}} \text{PS-NHC(=O)CH}_2\text{-CH}_2\text{-Lys-Pro conjugate}</math>  <math>\text{(3) + HS-Cys-Pro} \xrightarrow{\text{Neutral pH}} \text{PS-NHC(=O)CH}_2\text{-CH}_2\text{-Cys-Pro}</math> </p> </div> <b>(57) Abstract</b> <p>Novel immunogenic <math>\beta</math>-propionamido-linked polysaccharide- and N-propionamido-linked oligosaccharide-protein conjugates are provided as well as method of producing the conjugates. The conjugation procedure is simple, rapid, reproducible and applicable to a variety of polysaccharides or oligosaccharides derived from bacterial species, yeast, cancer cells or chemically synthesized. Vaccines and methods of immunization against infection or cancer using the immunogenic <math>\beta</math>-propionamido-linked polysaccharide- and <math>\beta</math>-propionamido-linked oligosaccharide-protein conjugates are also disclosed.</p>		

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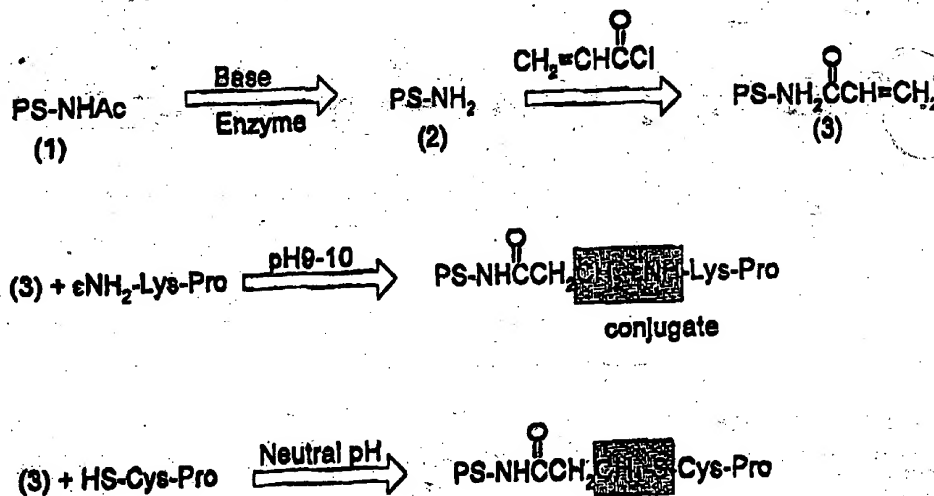
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(54) Title: IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE PROTEIN CONJUGATE USEFUL AS A VACCINE PRODUCED USING AN N-ACRYLOYLATED POLYSACCHARIDE



## (57) Abstract

Novel immunogenic  $\beta$ -propionamido-linked polysaccharide- and N-propionamido-linked oligosaccharide-protein conjugates are provided as well as method of producing the conjugates. The conjugation procedure is simple, rapid, reproducible and applicable to a variety of polysaccharides or oligosaccharides derived from bacterial species, yeast, cancer cells or chemically synthesized. Vaccines and methods of immunization against infection or cancer using the immunogenic  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-protein conjugates are also disclosed.

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**IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE  
PROTEIN CONJUGATE USEFUL AS A VACCINE PRODUCED USING AN N-  
ACRYLOYLATED POLYSACCHARIDE**

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**FIELD OF THE INVENTION**

The present invention relates to immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates and methods for producing the conjugates from bacteria, yeast, or cancer cells. The conjugates are useful as vaccines.

**BACKGROUND OF THE INVENTION**

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Bacterial infections caused by gram-positive bacteria such as Streptococcus, Staphylococcus, Enterococcus, Bacillus, Corynebacterium, Listeria, Erysipelothrix, and Clostridium and by gram-negative bacteria such as Haemophilus, Shigella, *Vibrio cholerae*, Neisseria and certain types of *Escherichia coli* cause serious morbidity throughout the world. This, coupled with the emerging resistance shown by bacteria to antibiotics, indicates the need for the development of bacterial vaccines. For example, streptococci are a large and varied genus of gram-positive bacteria which have been ordered into several groups based on the antigenicity and structure of their cell wall polysaccharide (26,27). Two of these groups have been associated with serious human infections. The group A streptococci cause a variety of infectious disorders including "strep throat", rheumatic fever, streptococcal impetigo, and sepsis. Group B streptococci are important perinatal pathogens in the United States as well as developing countries (37).

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Gram-negative bacteria are also a significant cause of disease. Until the recent development and use of polysaccharide-protein vaccines directed against *Haemophilus influenzae* type b bacteria (Hib), Hib bacterial infections were responsible for many cases of mental retardation in infants. *N. meningitidis* and *E. coli* K1 infections are responsible for neonatal meningitis. Strains of gram-negative bacteria, *E. coli*, have been linked to serious illness including death from eating meat tainted with *E. coli* strains.

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The polysaccharides have been used to elicit antibody responses to a variety of gram-negative and gram-positive bacteria, when conjugated to another immunogenic molecule such as a polypeptide or protein. Conjugation of the

polysaccharide or oligosaccharide to the polypeptide converts the immune response to the polysaccharide or oligosaccharide which is typically T-cell independent to one which is T-cell dependent.

The prior art discloses both direct coupling and indirect coupling of polysaccharides to proteins to form conjugates (summarized in Ref. (11) and U.S. Patent No. 5,306,492). Conjugation methods have included diazo coupling, thioether bond, amidation, reductive amination and thiocarbamoyl for coupling a polysaccharide to a protein carrier.

Geyer et al., Med. Microbiol. Immunol., 165: 171-288 (1979) describes conjugates of certain *Klebsiella pneumoniae* capsular polysaccharide fragments to a nitrophenyl-ethylamine linker by reductive amination and attachment of the derivatized sugar using azo coupling.

U.S. Patent No. 4,057,685 by McIntire describes a *Escherichia coli* lipopolysaccharide with reduced toxicity covalently coupled to a protein antigen by reaction with haloacyl halide.

U.S. Patent No. 4,356,170 by Jennings et al. describes the production of polysaccharide-protein conjugates by reductive amination.

U.S. Patent No. 4,673,574, 4,761,283 and 4,808,700 by Anderson describes the production of immunogenic conjugates comprising the reductive amination product of an immunogenic capsular polysaccharide fragment derived from the capsular polymer of *Streptococcus pneumoniae* or *H. influenzae* containing a reducing end prepared by means such as oxidative cleavage with periodate or by hydrolyses of a glycosidic linkage, with a bacterial toxin or toxoid as a protein carrier.

U.S. Patent No. 4,459,286 by Hillman et al. describes the preparation of a polysaccharide-protein conjugate by activation of the *H. influenzae* type b polysaccharide with cyanogen bromide, derivatization of the activated polysaccharide with the spacer molecule, 6-aminocaproic acid, and the conjugation of the major outer membrane protein of *Neisseria meningitidis* with a water soluble carbodiimide to form an amido type of linkage to the protein through a complex variety of linkages from the 6-aminocaproic acid spacer to the polysaccharide.

U.S. Patent No. 4,965,338 by Gordon describes the production of a water-soluble covalent polysaccharide-diphtheria toxoid conjugate, wherein a pure *H.*

*influenzae* type b polysaccharide is activated with cyanogen bromide and immediately mixed with diphtheria toxoid which has been derivatized with an ADH spacer.

U.S. Patent No. 4,663,160 by Tsay et al. describes a detoxified polysaccharide from a gram-negative bacteria covalently coupled to a detoxified protein from the same species of gram-negative bacteria by means of a 4-12 carbon moiety.

U.S. Patent No. 4,619,828 by Gordon et al describes conjugates between polysaccharide molecules from pathogenic bacteria such as *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Escherichia coli* and T cell dependent antigens such as diphtheria and tetanus toxoids.

U.S. Patent No. 4,711,779 by Porro et al describes glycoprotein conjugate vaccines having trivalent immunogenic activity comprising antigenic determinants from the capsular polysaccharides of a gram-positive bacteria, as well as either CRM<sub>197</sub>, tetanus toxoid, or pertussis toxin.

U.S. Patent No. 5,306,492 by Porro describes an oligosaccharide-carrier protein conjugate produced by reacting an oligosaccharide having a terminal reducing group with diaminomethane in the presence of pyridine borane such that reductive amination occurs, reacting the aminated oligosaccharide product with a molecule having two functional groups, and then reacting the activated oligosaccharide product with a carrier protein.

U.S. Patent No. 5,192,540 by Kuo et al describes a polysaccharide-protein conjugate comprising the reductive amination product of an oxidized polyribosyl-ribitol-phosphate polysaccharide fragment derived from the capsular polysaccharide of *Haemophilus influenzae* type b and the outer membrane protein of *Haemophilus influenzae* type b.

European publication No. EP 0747063 A2 describes a modified capsular polysaccharide containing multiple sialic acid derivatives and a heterobifunctional linker molecule linked to a carrier molecule. The linkers are used to N-alkylate up to about 5 sialic residues per polysaccharide. The remaining amino groups are then acylated with propionic or acetic anhydride.

More efficient, higher yielding and simpler means of obtaining purified immunogenic polysaccharide-protein conjugates for large-scale production of immunogenic polysaccharide-protein conjugate vaccines are desirable.



## SUMMARY OF THE INVENTION

The invention is an immunogenic  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-protein conjugate.

It is an object of this invention to provide a method for preparing immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates which provide advantages over currently employed methodologies. It is a further object of this invention to provide pharmaceutical compositions, vaccines and other immunological reagents derived from the immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates.

A method of preparing an immunogenic polysaccharide-protein conjugate is provided which comprises de-N-acetylation of a polysaccharide or an oligosaccharide by base or enzymatic hydrolysis followed by N-acryloylation of the N-deacetylated polysaccharide. The N-acryloylated polysaccharide is directly coupled to a carrier protein to form the immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugate.

Capsular and cell surface polysaccharides can be extracted according to this invention from either bacterial, yeast, or mammalian cell supernatants or directly from bacterial, yeast or mammalian cells by hydrolysis of the base labile bond that connects the polysaccharide to other cellular components or by enzymatic hydrolysis. A percentage of the N-acetyl groups removed by hydrolysis from the polysaccharide are replaced by N-acryloyl groups, which in turn, are directly coupled to protein to form the conjugate of the present invention.

An aspect of the invention provides oligosaccharides and polysaccharides that are directly coupled at multiple sites to protein(s).

Another aspect of the invention is a method of immunizing a mammal against bacterial or yeast infections or cancer, which comprises administration to the mammal an effective amount of the vaccine of the invention for prevention against infection from a disease causing organism or cancer.

An aspect of the invention is a method of eliciting the production of antibodies in mammals using the  $\beta$ -propionamido-linked polysaccharide-protein conjugates that protect the mammals against infection or disease.

Another aspect of the invention is immunoglobulin and isolated antibody elicited in response to immunization using  $\beta$ -propionamido-linked polysaccharide-protein conjugates. Such immunoglobulin and isolated antibody are useful as a therapeutics and as diagnostic reagents.

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1. Schematic of the method of making the immunogenic  $\beta$ -propionamido-linked polysaccharide-proteinconjugates.

#### DETAILED DESCRIPTION OF THE INVENTION

The invention is a novel polysaccharide-protein conjugate and oligosaccharide-protein conjugates useful as immunogens and vaccines against bacterial infections, yeast infections and as cancer therapeutics. Polysaccharides or oligosaccharides useful in forming immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates are derived from a source of polysaccharide or oligosaccharide which includes but is not limited to Gram (+) or Gram (-) bacteria, yeast, cancer cells or cancerous tissues and the like in which the polysaccharide or oligosaccharide serves as a virulence factor for the cell in evading host defense mechanisms. The polysaccharide-protein conjugates of the present invention are formed by direct coupling of the N-acryloylated polysaccharide with a protein by a Michael-type addition of nucleophilic sites on proteins.

Polysaccharides or oligosaccharides may be obtained from a variety of sources including gram-negative, gram-positive bacteria, yeast, cancer cells or recombinant forms of each using base or enzymatic hydrolysis of the bond that attaches the polysaccharide or oligosaccharide to the cellular components. Polysaccharide or oligosaccharide may be extracted from the organism or cell by contacting the organism or cell or a solution containing fragments of the organism or cell with an base or enzyme. Polysaccharide or oligosaccharide may then be recovered after basic or enzymatic hydrolysis by a variety of methods. Non-limiting examples of gram-positive bacteria and recombinant strains thereof for use according to this invention are Streptococci, Staphylococci, Enterococci, Bacillus, Corynebacterium, Listeria, Erysipelothrix, and Clostridium. Specifically, the use of Streptococci is more preferred and the use of group B Streptococci types Ia, Ib, II, III, IV, V, and VIII is most preferred. Non-limiting examples of gram-negative bacteria

and recombinant strains thereof for use with this invention include *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Specifically, the use of *H. influenzae* type b, *N. meningitidis* types B, C, Y and W135, *E. coli* K1, and *E. coli* K92 are more preferred. Examples of yeast for use in the present invention include but are not limited to *Cryptococcus neoformans*. Examples of cancer cells or cancerous tissue for use in the present invention include but are not limited to small cell lung carcinoma, neuroblastomas, breast cancer, colon carcinoma, and the like.

A wide variety of conditions can be used for hydrolysis of the polysaccharide or oligosaccharide in either aqueous or organic solvent according to the invention by methods known in the art. The extent to which N-acetyl bonds of the carbohydrates are hydrolyzed can be controlled by the reaction conditions. In one embodiment, at least about 50% of the N-acetyl groups are removed by hydrolysis, preferably about 50% to about 100% are removed, more preferably about 90% or more of the native N-acetyl groups are removed. In a particular embodiment, about 95% or more of the N-acetyl groups are hydrolyzed from the polysaccharide by treatment with a hydrolysis reagent.

Capsular polysaccharides amenable to base extraction are those polysaccharides that lack any base-labile substituent that cannot be replaced, such as O-acetyl groups critical to immunogenicity. Other capsular polysaccharides amenable to base extraction are those lacking a phosphodiester bond and those lacking 4-linked uranic acid residues.

In a preferred embodiment for base hydrolysis, the CPS are extracted from group B Streptococci (GBS). In a most preferred embodiment the CPS are extracted from GBS types Ia, Ib, II, III, V and VIII.

In another preferred embodiment for base hydrolysis, the CPS are extracted from *S. pneumoniae*. In a more preferred embodiment for base hydrolysis the CPS are extracted from *S. pneumoniae* types III, IV and XIV.

In another preferred embodiment for base hydrolysis, the CPS are extracted from *Neisseria* or *Escherichia* bacteria. In a more preferred embodiment for base extraction, the CPS are extracted from *Neisseria meningitidis* types B, C, Y or W135, *Escherichia coli* K1 or *Escherichia coli* K92.

Polysaccharides amenable to enzymatic de-acetylation are those polysaccharides that lack any enzyme-labile substituent critical to immunogenicity in which the substituent cannot be replaced or substituted by an immunogenic moiety, these polysaccharides include but are not limited to GBS and the like.

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**A. Preparation of the N-acryloylated polysaccharides**

**1. Deacetylation of Polysaccharides**

**a). Starting Materials**

Polysaccharide or oligosaccharide may be obtained using base hydrolysis or enzymatic hydrolysis from concentrated bacterial, yeast, mammalian  
10 cells or recombinant forms of these cells or from supernatants from homogenized cells or from conditioned medium using standard methods known in the art. The polysaccharide or oligosaccharide may be isolated and purified by standard methods known in the art. Isolated and purified polysaccharide or oligosaccharide from commercial sources may also be used as starting material.

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Methods for isolation of the polysaccharide depend on the particular polysaccharide being used. A common method is the use of ionic detergent to complex with a charged polysaccharide. The complex is precipitated and isolated. The complex is then dissolved in a solution of high ionic strength such as calcium chloride and the polysaccharide is then precipitated with ethanol

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The isolated and purified polysaccharides and oligosaccharides obtained for use in this invention preferably contain less than 1% nucleic acid and protein impurities for human use. Purities of 80-100% carbohydrate are often observed after purification due to the presence of inorganic salts.

**b). Base Hydrolysis**

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To remove the N-acetyl groups the purified polysaccharides or oligosaccharides can be treated with bases. Non-limiting examples of bases which may be used according to this invention are NaOH, KOH, LiOH, NaHCO<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub>, KCN, Et<sub>3</sub>N, NH<sub>3</sub>, H<sub>2</sub>N<sub>2</sub>H<sub>2</sub>, NaH, NaOMe, NaOEt or KOtBu. Bases such as NaOH, KOH, LiOH, NaH, NaOMe or KOtBu are most effectively used in a range of  
30 0.5 N - 5.0 N. Bases such as NaHCO<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub> and KCN can be used in concentrations as high as their solubilities permit. Organic bases such as Et<sub>3</sub>N can be used at medium to high (50-100%) concentrations as long as there is an agent such as

water or alcohol to effect the hydrolysis. Bases such as  $\text{NH}_3$  or  $\text{H}_2\text{N}_2\text{H}_2$  can be used at nearly any concentration including 100%. Solvents such as water, alcohols (preferably  $\text{C}_1\text{-C}_4$ ), dimethylsulfoxide, dimethylformamide or mixtures of these and other organic solvents can be used. Base solutions comprising water are most preferred.

The most effective pH range for removal of N-acetyl groups from the polysaccharide or oligosaccharide is from about 9 to about 14 with the optimal pH being around 12. The N-deacetylated polysaccharide thereafter is purified from residual reagents by ultrapurification using membranes or dialysis by standard methods known in the art.

#### c). Enzymatic Hydrolysis

The enzyme, N-deacetylase may be used to enzymatically removed N-acetyl groups from a polysaccharide or oligosaccharide. In one embodiment, an N-deacetylase enzyme useful in removal of N-acetyl residues from polysaccharides or oligosaccharides is described in Refs 47, 48 and 49. In enzymatic hydrolysis, the polysaccharide or oligosaccharide and deacetylase enzyme are mixed with an appropriate enzyme buffer system under appropriate pH and temperature conditions and allowed to react for a period sufficient for removal of N-acetyl groups. In one embodiment, polysaccharide and deacetylase enzyme are mixed with an appropriate enzyme buffer system, for example, 50 mM MES, 10 mM  $\text{MnCl}_2$ , pH 6.3 at  $37^\circ\text{C}$  for 60 minutes for formation of N-deacetylated polysaccharide. The reaction is stopped using an appropriate stopping solution for example 1 M monochloroacetic acid, 0.5 M NaOH, 2 M NaCl, or by dilution using an appropriate buffer solution.

#### 2. N-Acryloylation of the Polysaccharide

The alkaline or enzymatic hydrolysis of the polysaccharide or oligosaccharide results in the removal of N-acetyl groups from sialic acid and amino sugar residues of the polysaccharides or oligosaccharides. After hydrolysis, the polysaccharide or oligosaccharide is N-acryloylated to the extent desired by using a variety of acryloylating agents.

In one embodiment, the method comprises adding an acryloylating reagent to N-acryloylate an N-deacetylated polysaccharide or oligosaccharide. Examples of acryloylation reagents include but are not limited to acryloyl chloride,

acryloyl anhydride, acrylic acid and a dehydrating agent such as DCC,  $\text{CH}_2\text{CHCOCN}$  the like, used in excess at a concentration of about 1 M. In a method of N-acryloylation of an N-deacetylated polysaccharide, the pH is adjusted and maintained at about 9 to about 11, preferably about pH 10 during the reaction. The temperature during reaction is about 2°C to about 8°C, preferably about 4°C. The reaction is carried out over a period of about 1 hour. The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater.

#### B. Preparation of $\beta$ -propionamido-linked polysaccharide-protein conjugates

The polysaccharide or oligosaccharide of this invention may be used to elicit antibody responses to a variety of gram-negative and gram-positive bacteria, yeast and cancers in an individual when conjugated to another immunogenic molecule such as a polypeptide or protein. Conjugation of the polysaccharide or oligosaccharide to the polypeptide converts the immune response to the polysaccharide or oligosaccharide which is typically T-cell independent to one which is T-cell dependent. Accordingly, the size of the polypeptide is preferably one which is sufficient to cause the conversion of the response from T-cell independent to T-cell dependent. It may be useful to use smaller polypeptides for the purpose of providing a second immunogen. The size of the protein carrier is typically from about 50,000 to about 500,000 M.W.

Preferred carrier proteins include, but are not limited to, tetanus toxoid, diphtheria toxoid, cholera toxin subunit B, *Neisseria meningitidis* outer membrane proteins, pneumolysin, C- $\beta$  protein from group B Streptococcus, non-IgA binding C- $\beta$  protein from group B Streptococcus, *Pseudomonas aeruginosa* toxoid, pertussis toxoid, synthetic protein containing lysine or cysteine residues, and the like. The carrier protein may be a native protein, a chemically modified protein, a detoxified protein or a recombinant protein. Conjugate molecules prepared according to this invention, with respect to the protein component, may be monomers, dimers, trimers and more highly cross-linked molecules.

This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide. The size of the polysaccharide or

oligosaccharide may vary greatly. One or a multiplicity of polysaccharides or oligosaccharides may cross-link with one or a multiplicity of protein. The conjugates of the present invention are preferably lattice structures. The points of attachment are between lysine or cysteine residues of the protein and the N-acryloyl groups of the polysaccharide or oligosaccharide.

In one method of forming an immunogenic polysaccharide-protein conjugate, an isolated polysaccharide (glycosaminoglycan) containing free amino groups or N-acyl groups (e.g. N-acetyl groups) in the sugar residues that constitute its repeating unit, is first treated hydrolyzed using base or enzyme to remove part or all of its N-acyl groups. The free amino groups are then N-acylated with an N-acryloylating reagent to form the N-acryloylated polysaccharide described above. The N-acryloylated polysaccharide is then directly coupled to protein under optimum conditions of pH, temperature and time to form an immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugate.

In one embodiment, the method of conjugation is conducted at a pH above 9.0, preferably a pH of about 9.0 to about 10.0 for optimal reactivity of  $\epsilon$ -free amino groups of lysine residues on the protein. In another embodiment, the method of conjugation is conducted at a neutral pH of about 7.0 for optimal reactivity of thiol (SH) groups of cysteine residues of the protein. The selection of pH for conducting the method of conjugation may be based on the number of reactive groups in a particular carrier protein. For example, a method using a protein composed of more reactive lysine residues as compared to cysteine residues is preferably conducted at a basic pH. A method of conjugation using a protein composed of more reactive cysteine residues as compared to lysine residues is preferably conducted at about a neutral pH.

The conjugation reaction may be conducted in buffered reagents including but not limited to a buffered reagent including carbonate/bicarbonate, borate buffer, phosphate and the like. The temperature of the conjugation reaction is at least about 25 °C, preferably about 37 °C, for a period of preferably about 24 hours. The key reaction involves a 1,4-conjugate addition (Michael-type addition) of nucleophilic cysteine thiol groups or lysine  $\epsilon$ -NH<sub>2</sub> groups on proteins with N-acryloylated sugar residues as described by Romanowska et al (46) which are present in the repeating-

unit of the polysaccharide as shown in Figure 1. The resulting  $\beta$ -propionamido-linked polysaccharide-protein conjugate has a polysaccharide to protein ratio of about 0.1 to about 0.6.

The glycosyl residues of the polysaccharide having N-acyl groups  
5 amenable to direct conjugation with cysteine and/or lysine residues on protein include, but are not limited to, glucosamine, galactosamine, mannosamine, fucosamine, sialic acids and the like. The polysaccharide may be derived from natural sources such as bacteria, yeast or cancer cells or from synthetic sources. Synthetic sources include chemical synthesis, enzymatic synthesis and chemoenzymatic synthesis. The  
10 synthesis may be *de novo* synthesis or the modification of natural carbohydrates. Naturally isolated carbohydrates can be modified by altering functional groups on carbohydrate residues or by the addition or removal of carbohydrate residues.

The polysaccharide or oligosaccharide for use in preparing the  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-  
15 protein conjugates of the present invention may vary in size for conjugation with a carrier protein. As defined herein, an oligosaccharide for use in the present invention comprises at least 10 sugar residues and preferably from 10 to about 50 sugar residues. A polysaccharide, as defined herein, is greater than 50 sugar residues and may be as large as about 600 or greater residues. In some cases, large constructs are desirable for  
20 enhancement of immunogenicity. The methods of this invention provide for the use of very large polysaccharides because many reactive sites can be introduced into a single polysaccharide. Another advantage of this method over the prior art is that the polysaccharide or oligosaccharide is not altered at a charged functional group which often interact with/or form part of the epitope crucial for immunity.



### C. Vaccines

This invention is also directed to vaccine preparations. According to this invention, the isolated  $\beta$ -propionamido-linked polysaccharide-protein conjugates described above may be used as an antigen to generate antibodies that are reactive against the polysaccharide or oligosaccharide and hence reactive against the organism or cell from which the polysaccharide or oligosaccharide was isolated. The vaccines of the present invention may be a combination or multi component vaccine further comprising in combination with the  $\beta$ -propionamido-linked polysaccharide-protein conjugate other components, including but not limited to Diphtheria-Tetanus-Pertusis (DTP), Tetanus-Diphtheria (Td), DTaP, a DTaP-Hib vaccine, a DTaP-IPV-Hib vaccine, and the like and combinations thereof, to provide a multifunctional vaccine useful in immunizing against a variety of diseases causing organisms or disease causing cells.

The vaccines of this invention may provide active or passive immunity. Vaccines for providing active immunity comprise an isolated and purified N-acryloylated polysaccharide or oligosaccharide conjugated to at least one antigenic peptide.

### D. Pharmaceutical compositions

The pharmaceutical compositions of this invention may comprise at least one polysaccharide-protein conjugate and pharmacologically acceptable carriers such as saline, dextrose, glycerol, ethanol or the like. In another embodiment the pharmaceutical composition comprises another immunogenic moiety, such as a peptide, or compositions comprising antibodies elicited by one of the CPS of this invention. The composition may also comprise adjuvants to enhance the immunological response of the recipient. Such adjuvants may be aluminum based such as alum or long chain alkyl adjuvants such as stearyl tyrosine (see U.S. Serial No. 583,372, filed 9/17/90; European Patent, EP 0 549 617 B1; Moloney et al. U.S. Patent No. 4,258,029), muramyl dipeptide (MDP) or derivative thereof, monophosphoryl lipid A (MPL), saponin (Quil-A) and the like. See also Jennings, et al. U.S. Patent No. 5,683,699 and Paoletti, et al. J. Infectious Diseases 1997; 175:1237-9. The pharmaceutical composition may further comprise one or more additional immunogens including but not limited to Diphtheria-Tetanus-Pertusis (DTP), Tetanus-

Diphtheria (Td), DTaP, DTaP-Hib, DTaP-IPV-Hib, and the like and combinations thereof. These pharmaceutical compositions are particularly useful as vaccines.

For eliciting passive immunity, the pharmaceutical composition may be comprised of polyclonal antibodies, or monoclonal antibodies, their derivatives or fragments thereof and recombinant forms thereof. The amount of antibody, fragment or derivative will be a therapeutically or prophylactically effective amount as determined by standard clinical techniques.

The pharmaceutical preparations of this invention may be introduced to an individual by methods known to be effective in the art. Intradermal, intraperitoneal, intravenous, subcutaneous, intramuscular, oral and intranasal are among, but not the only, routes of introduction.

The compositions of the invention may comprise standard carriers, buffers or preservatives known to those in the art which are suitable for vaccines including, but not limited to, any suitable pharmaceutically acceptable carrier, such as physiological saline or other injectable liquids. Additives customary in vaccines may also be present, for example stabilizers such as lactose or sorbitol and adjuvants to enhance the immunogenic response such as aluminum phosphate, hydroxide, or sulphate and stearyl tyrosine. The vaccines produced according to this invention may also be used as components of multivalent vaccines which elicit an immune response against a plurality of infectious agents.

Vaccines of the present invention are administered in amounts sufficient to elicit production of antibodies as part of an immunogenic response. The vaccine can be used parenterally to produce IgG and IgM antibodies or it can be delivered to the mucosal membranes to elicit IgA antibodies on the surface of tissues. Dosages may be adjusted based on the size, weight or age of the individual receiving the vaccine. The antibody response in an individual can be monitored by assaying for antibody titer or bactericidal activity and boosted if necessary to enhance the response. Typically, a single dose for an infant is about 10  $\mu$ g of conjugate vaccine per dose or about 0.5  $\mu$ g-20  $\mu$ g/kilogram. Adults receive a dose of about 0.5  $\mu$ g-20  $\mu$ g/kilogram of the conjugate vaccine. For the CPS-protein conjugate vaccine, a typical dose is about 25  $\mu$ g of each individual CPS per dose. That is, a vaccine against group B streptococcus may comprise 25  $\mu$ g of each of the CPS from each of the nine serotypes.

### E. Antibodies

Antibodies directed against the polysaccharide may be generated by any of the techniques that are well known in the art. According to one approach, the antibodies may be generated by administering an isolated immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugate into a host animal. The host animal may be, but is not limited to, rat, mouse, rabbit, non-human primate, or a human. Preferably, the host is human. In one embodiment, immunological responses may be increased by the use of adjuvants which are known in the art

Monoclonal antibodies directed against the polysaccharide may also be prepared by any of the techniques that are well known in the art. According to one method, cultures of hybridoma cell lines are used (Kohler and Milstein (1975) *Nature* 256:495-497). Monoclonal antibodies directed against the polysaccharide may be human monoclonal antibodies, chimeric monoclonal antibodies or humanized monoclonal antibodies made by any of the techniques that are well known in the art. According to one approach, chimeric monoclonal antibodies may be generated that have a non-human (e.g. mouse) antigen-binding domain combined with a human constant region. (Takeda et al. (1985) *Nature* 314:452). Humanized antibodies can be generated according to the procedures of Queen et al., U.S. Patent No. 5,585,089 and U.S. Patent No. 5,530,101. Single chain antibody may be constructed by methods known in the art (U.S. Patent No. 4,946,778; Davis, G.T. et al 1991 *Biotechnology* 9:165-169; Pluckthun, A. 1990 *Nature* 347:497-498). Constant region domains of the antibody may be modified by procedures known in the art (WO 89/07142)

Antibodies directed against the polysaccharide or oligosaccharide may be purified by any of the techniques that are well known in the art including, but not limited to immunoabsorption or immunoaffinity chromatography, or other chromatographic methods (e.g. HPLC). Antibodies may also be purified as immunoglobulin fractions from serum, plasma or cell culture medium.

Antibody molecules of this invention may be intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of an immunoglobulin molecule, for example Fab fragments, that contain the antigen binding site. The antibody molecules may be of any class including IgG, IgM, and IgA.

Fragments of antibodies directed against the CPS may be generated by any of the techniques that are well known in the art. (Campbell (1985) Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 13, Burdon, et al. (eds.), Elsevier Science Publishers, Amsterdam).

5           The antibody or antigen or antigen binding fragment thereof is useful as a therapeutic in providing passive protection against diseases caused by Gram (+), Gram (-) bacteria or yeasts. The antibody or antigen binding fragment thereof are also useful as a diagnostic reagent in standard immunoassays for the detection and/or identification of bacteria, yeast or cancer cells. The antibody may be supplied in kit  
10   form alone or with standard reagents for immunoassays.

          In another embodiment of this invention, antibodies directed against the polysaccharide or oligosaccharide of this invention may be used as a pharmaceutical preparation in a therapeutic or prophylactic application in order to confer passive immunity from a host individual to another individual (i.e., to augment an individual's  
15   immune response against gram-negative or gram-positive bacteria or yeast or to provide a response in immuno-compromised or immuno-depleted individuals including AIDS patients). Passive transfer of antibodies is known in the art and may be accomplished by any of the known methods. According to one method, antibodies directed against the conjugates thereof of this invention are generated in an  
20   immunocompetent host ("donor") animal, harvested from the host animal, and transfused into a recipient individual. For example, a human donor may be used to generate antibodies reactive against the polysaccharide-protein conjugate of this invention. The antibodies may then be administered in therapeutically or prophylactically effective amounts to a human recipient in need of treatment, thereby  
25   conferring resistance in the recipient against bacteria which are bound by antibodies elicited by the polysaccharide component. (See Grossman, M. and Cohen, S.N., in "Basic and Clinical Immunology", 7th Ed., (Stites, D. P. and Terr, A. T. eds., Appleton & Lange 1991) Chapter 58 "Immunization".)

          In certain cases the polysaccharide used with this invention may induce  
30   antibody which is cross-reactive with other pathogenic organisms and thus have ability in protecting against infection by these other bacteria.

#### F. Diagnostic kits

In another embodiment, the CPS of this invention or derivatives or fragments thereof may be provided in diagnostic kits to indicate the presence of antibodies directed against bacteria, yeast or cancer cells. The presence of such antibodies can indicate prior exposure to the pathogen, and predict individuals who may be resistant to infection. The diagnostic kit may comprise at least one of the CPS of this invention or derivatives or fragments thereof, alone or conjugated to protein, and suitable reagents for the detection of an antibody reaction when the modified CPS or derivatives or fragments are mixed with a sample that contains antibody directed against gram-negative, gram-positive bacteria, yeast or cancer cells or cancer tissue. An antibody reaction may be identified by any of the methods described in the art, including but not limited to an ELISA assay. Such knowledge is important, and can avoid unnecessary vaccination.

Alternatively, the diagnostic kit may further comprise a solid support or magnetic bead or plastic matrix and at least one of the CPS of this invention or derivatives or fragments thereof.

In some cases, it may be preferred that the CPS or derivatives or fragments are labeled. Labeling agents are well-known in the art. For example, labeling agents include but are not limited to radioactivity, chemiluminescence, bioluminescence, luminescence, or other identifying "tags" for convenient analysis. Body fluids or tissues samples (e.g. blood, serum, saliva) may be collected and purified and applied to the diagnostic kit. The CPS, derivatives or fragments may be purified or non-purified and may be composed of a cocktail of molecules.

Solid matrices are known in the art and are available, and include, but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate, or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well micro titer plates, test tubes and Eppendorf tubes. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

All publications, patents and articles referred to herein are expressly incorporated herein *in toto* by reference thereto. The following examples are

presented to illustrate the present invention but are in no way to be construed as limitations on the scope of the invention. It will be recognized by those skilled in the art that numerous changes and substitutions may be made without departing from the spirit and purview of the invention.

### EXAMPLE 1

#### **Preparation of $\beta$ -Propionamido-Linked Polysaccharide-Protein Carrier Conjugates**

The following non-limiting examples describe the preparation of a series of clinically relevant polysaccharide-protein conjugates for vaccines against *Streptococcus pneumoniae* (type 14), Group B *Streptococcus* (GBS) type III and type II, and *E.coli* K1. All of the above polysaccharides used in this example are glycosaminoglycans that contain N-acetyl groups in one or more of the glycosyl residues that are constituents of their structural repeating-units.

##### **A. Depolymerization of type 14 pneumococcal polysaccharide**

To increase its solubility the polysaccharide was first partially depolymerized by sonication. 200 mg of *Pneumococcal polysaccharide* type 14 (Lot NO 2020510, American Type Culture Collection) was dissolved in 20 ml of PBS and sonicated for 4 hours at 0°C with a Branson Sonifier Model 450. The resulting polysaccharide was dialyzed and lyophilized and then sized through a superdex 200 column equilibrated with phosphate buffered saline (PBS). Peak fractions were pooled and then dialyzed against d.i. water with Spectra/Por® Membrane MWCO:3,500. A yield of 157.5mg solid was obtained after lyophilization. The sonicated polysaccharide had an average molecular weight of about 50,000 as measured by SEC-MALLS with the miniDAWN (Wyatt Technology Corp., Santa Barbara, CA).

##### **B. De-N-Acetylation of type 14 pneumococcal polysaccharide**

100 mg of sized type 14 pneumococcal polysaccharide was dissolved in 10 ml of 2N NaOH and then 10 mg of NaBH<sub>4</sub> was added to the reaction mixture. This mixture was heated at 100 °C for one hour and then cooled to room temperature. The N-deacetylated component was dialyzed against d.i. water with a Spectra/Por® Membrane Membrane MWCO:3,500 and lyophilized to give 84 mg of white solid. The N-deacetylated polysaccharide was analysed by H<sup>1</sup>-NMR at 500 MHz and was found to contain less than 5 percent residual N-acetyl groups.

### C. N-Acryloylation of the N-deacetylated type 14 Pneumococcal polysaccharide

84 mg of N-deacetylated type 14 Pneumococcal polysaccharide was dissolved in 4.2 ml of d.i. water. The solution, in an ice bath, was adjusted to pH 10 with 2 N NaOH. Then 420  $\mu$ l of 1:1 v/v acryloyl chloride : dioxane was added and adjusted to pH 11 with 2 N NaOH. The reaction was allowed to stand for an additional hour at pH 11 to ensure the complete hydrolysis of esters which may have formed as a result of O-acylation. The solution was dialyzed and lyophilized to give 42 mg of dry powder. After analysis by 500 MHz  $H^1$ -NMR the polysaccharide was found to be over 95 percent N-acryloylated.

### D. Coupling of the type 14 N-acryloylated pneumococcal polysaccharide to tetanus toxoid monomer

22 mg of the type 14 N-acryloylated pneumococcal polysaccharide was dissolved in 1.1 ml of Carbonate/Bicarbonate pH 9.5 buffer. Tetanus toxoid monomer 22 mg was added to the reaction mixture. The reaction mixture was incubated overnight at 37 °C. The progress of the conjugation was analyzed with a Biologic system (Bio-Rad) equipped with a superose 12 column. Conjugation of polysaccharide to tetanus toxoid was indicated by the progressive increase in a peak, monitored by measurement of UV absorbance at 280 nm, eluting in the void volume of the column. After conjugation was complete, the solution was neutralized to pH 7 with 0.1N HCl and then dialyzed against PBS. The conjugate was purified by passage over a 1.6x60cm column of Superdex 200 PG (Pharmacia) and eluted with PBS containing 0.01% thimerosal. Fractions corresponding to the void-volume peak were pooled. Carbohydrate and protein content in the conjugate were estimated by the phenol-sulfuric assay of Dubois et al. (51) and the Coomassie assay of Bradford (9).

Similar methods were used for GBS type II, type III as well as for the *E. coli* K1 and meningococcal C polysaccharides. The reaction conditions for each of these polysaccharides are tabulated below.

**TABLE 1**

**E. De-N-Acetylation of GBS Type II and Type III Polysaccharide**

	PS size* (kD)	PS in mg	NaOH	NaBH <sub>4</sub>	Temp.	Reaction time	yield
GBSP II	250	63 mg	6 mL	12 mg	110 °C	6 h	63 mg
GBSP III	110	50 mg	5 mL	10 mg	110 °C	6 h	55 mg

5 \*Determined by SEC-MALLS

**TABLE 2**

**N-Acryloylation of GBS Type II and Type III polysaccharide**

	PS amount in mg	d.i. water	1:1 v/v acryloyl chloride:dioxane	yield
GBSP II	60	3 ml	300 µl	60 mg
GBSP III	55	2.75 mL	275 µl	55 mg

**TABLE 3**

**Coupling of the GBS II and GBS III polysaccharide to Tetanus Toxoid Monomer**

	PS in mg	TT in mg	Carbo/Bicarb buffer pH 9.5	temperature	Incubation time
GBSP II	10	10	0.5 mL	37 °C	overnight
GBSP III	10.52	9.52	0.5 mL	37 °C	overnight

10

**F. De-N-Acetylation of K1 polysaccharide**

300 mg of K1 PS was dissolved in 15 mL of 2.0 N NaOH solution to which 150 mg of sodium borohydride was added. The solution was heated at 110 °C for 6 hours, cooled down to room temperature and diluted with a 20-fold volume of deionized water. After diafiltration through an Amicon YM3 membrane with deionized water, the solution was lyophilized yielding 255 mg of N-deacetylated K1 PS. H<sup>1</sup>-NMR at 500 MHz confirmed that complete N-deacetylation occurred.

20

**G. N-Acryloylation of K1 polysaccharide**

To a 10 mL deionized water solution containing 250 mg of de-N-acetylated K1 PS, cooled in an ice bath, was added dropwise acryloyl chloride



(Aldrich, Milwaukee, WI) solution, prepared by combining 1 mL of acryloyl chloride with a 1 mL of dioxane. The pH of the solution was maintained between 7.0 and 10.5 by the addition of 2 N sodium hydroxide solution. After completion of the addition, the pH was raised to 13 and maintained between 12.9 to 13.1 for 1 hour at room temperature. The pH of the solution was adjusted to 9.5 by the dropwise addition of 1 N HCL. The solution was diafiltrated with an Amicon YM3 membrane in a stircell with deionized water. The retentate was lyophilized to dryness, and the material (N-Acryloyl K1 PS) was stored at in a desiccator in a -20 C freezer. H-NMR at 500 MHz indicated that complete N-acryloylation took place during the reaction.

#### 10 H. $\beta$ -Propionamido-Linked K1-rPorB conjugate (K1-rPorB I)

A solution containing 8.4 mg of N-Acryloyl K1 PS and 4.0 mg of recombinant *Neisseria meningitidis* PorB in 0.3 mL of 0.2 M borate, 0.05% Zwittergen™ 3,14 (Boehringer Mannheim) pH 9.5 was incubated at 37° C for 3 days. The conjugate was purified by size exclusion chromatography through a Superdex 200 preparative grade column, and eluted with PBS containing 0.01% thimerosal. The fractions of uv-280 nm active signal eluting at or close to the void volume of the column were pooled and stored in the refrigerator. The conjugate was analysed for sialic acid and protein content by the resorcinol and Coomassie protein assays respectively.

20

#### I. Preparation of Thiolated rPorB

To one ml of rPorB porin solution at a conc of 10 mg/ml in 0.25 M HEPES buffer of pH 8.5 containing 0.25 M sodium chloride and 0.05% zwittergent 3-14 was added 0.2 ml of 0.05 M N-succinimidyl 3-[2-pyridyldithio]propionate solution. The solution was mixed well and allowed to sit at RT for one hour. To the solution was added 0.06 ml of 1 M dithiothreitol solution in the same buffer. The solution was again mixed well and allowed to sit at RT for an additional two hours. The solution was diluted with 1.3 ml of 0.25 M HEPES buffer of pH 7.0 containing 0.25 M sodium chloride and 0.05% zwittergent 3-14 and loaded onto a Pharmacia PD-10 desalting column which had been pre-equilibrated with the same buffer. The column was eluted with the same buffer, and eluate was collected and concentrated with an

30

TABLE 4

## Analytical Data for the Conjugates Described Above

	Protein Conc. μg/mL	PS Conc. μg/mL	Percent PS in conjugate
Pn14-TT (3)	547	293 (1)	35
GBSII-TT(3)	377	160 (2)	30
GBSIII-TT (3)	365	115 (2)	24
K1-rPorB I (3)	147	17 (2)	10
K1-rPorB II (4)	406	41 (2)	9
K1-S-rPorB (3)	188	25 (2)	12
GCMP-S-rPorB (3)	200	43 (2)	18

5

- (1) Total carbohydrate Dubois assay  
 (2) resorcinol sialic assay  
 (3) Prepared by direct coupling of the N-Acryloylated polysaccharide and the corresponding carrier protein  
 10 (4) Control conjugate prepared by reductive amination of a periodate-oxidized N-Acryloylated K1 PS with rPorB

## EXAMPLE 2

15

### Immunogenicity and Potency of the $\beta$ -Propionamido-Linked Polysaccharide-Protein Carrier Conjugates

#### Preclinical evaluation of the conjugates in mice

- Immunoassays: Serum antibody to each polysaccharide conjugate was  
 20 measured by ELISA. The human serum albumin (HSA) (Sigma, St Louis, MO) conjugates used for ELISA assays were prepared by reductive amination. The oxidized polysaccharides were added to HSA followed by reductive amination with NaCNBH<sub>3</sub>. The conjugates were isolated by gel filtration chromatography, and stored freeze-dried at -70 °C. PS-specific antibody titers were determined by an ELISA as follows.  
 25 Polystyrene, 96-well, flat-bottom microtiter plates (NUNC Polysorb) (Nuñc, Naperville, IL) were coated with PS-HSA conjugates in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.5 ) at 0.25 μg/well (100μL/ well) by incubating for 1 hour at 37 °C, followed by a PBS-Tween (0.05% v/v Tween 20 in PBS) wash (5 times). All subsequent incubations were conducted at room temperature. PBS-Tween  
 30 was used for all required washes. The coated plates were then blocked with PBS-BSA

(0.5% w/v bovine serum albumin in PBS) for IgG ELISAs or 0.1% w/v Carnation nonfat dry milk for IgM ELISAs at 0.15 mL / well for 1 hour, followed by a wash. Sera were diluted 2-fold, in duplicate, in the plate at 100  $\mu$ L/ well and incubated for 1 hour, followed by a wash. Antibody conjugate (peroxidase-labelled goat anti-mouse (Kirkegaard & Perry Lab, Gaithersburg, MD) was added at 100  $\mu$ L/ well and incubated for 30 minutes, followed by a wash. A 1:1 dye and substrate solution (Kirkegaard & Perry TMB) and peroxide was added at 0.05mL/ well and incubated for 10 minutes. The peroxidase reaction was then stopped with 1 M  $H_3PO_4$  at 0.05 mL/ well, and the plate was read on a Molecular Devices Emax microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 450 nm, using 650 nm as a reference wavelength. Background absorbances were determined in several no-serum control wells and averaged for each plate. For each serum dilution, the average background absorbance was subtracted, and then duplicate serum absorbance values were averaged. A modified Scatchard plot was used for the subsequent data analysis, where the absorbance (y-axis) was plotted against the absorbance times the reciprocal dilution (x-axis) (ref). Under conditions allowing equilibrium and antibody excess, a straight line was obtained for each serum dilution series; this line was extrapolated to the x-axis for the determination of an antibody titer. A positive control serum, with a previously determined antibody titer, was used on each plate in order to provide a reference to which all sera were standardized, minimizing plate to plate and day to day variations. The results of these assays are shown in Tables 5, 6 and 7.

Opsonophagocytic assays (OP) : The opsonic activity of mice antisera to the Streptococcal B (GBS) and Pneumococcal conjugates was tested in an *in vitro* opsonophagocytic killing assay using the human promyelocytic leukemia HL-60 cell line (ATCC No. CCL 240). Briefly, 200 cfu of GBS type III strain M781 cells or pneumococcal type 14 strain were mixed in equal volume with serum antibodies and incubated under shaking 15 minutes at 35 °C in a 5%  $CO_2$  incubator. Baby rabbit complement and HL-60 cells ( $5 \times 10^5$ ) cultured 5 days in the presence of 90 mM DMF were added to the mixture and incubated at 37 °C for 1 hour under shaking. Aliquots were removed for quantitative culture. Titers were determined by extrapolating the antibody dilution corresponding to fifty percent live bacteria. The results of these

assays are shown in Table 5 for the pneumococcal type 14 conjugates and in Table 6 for the GBS type III conjugates.

*Serum bactericidal assay (SBA)* : Antibody-dependent complement-mediated bactericidal activity was measured in terms of the bactericidal titer, or reciprocal dilution, that provided 50% killing of the targeted bacteria. The complement in all sera was first incubated at 56 °C for 30 min. Then a 2-fold dilution series was established for each serum with GBSS in sterile 96-well U-bottom microtiter plates (Sigma), giving a final volume of 50 µL/ well. Infant rabbit serum complement (Pel-Freez, Brown Deer, WI) was diluted 1:1 with the working concentration of GBM bacteria (serotype 15 strain, 44/76) or Group C meningococcal C11 reference strain and 50 µL was added to each well containing the diluted serum, giving a final reaction mixture volume of 100 µL/ well. This reaction mixture, which contained 50% serum (heat-inactivated and diluted), 25% rabbit serum complement, and 25% bacteria (at working concentration), was incubated in a humidified incubator at 37 °C with 5% CO<sub>2</sub> for 60 min on a microtitration plate shaker (LKB-Wallac; pharmacia Biotech) at the fast speed.

All wells were then plated on chocolate Agar by spreading 30 µL/ plate. Time zero bacterial samples were also plated. All plates were incubated overnight, as before. The colony-forming units (cfu) were then counted with an automated colony counter from Imaging Products International (Chantilly, VA), taking an average of three readings per plate. The reciprocal dilution, or titer, that gave 50% killing was read directly from a graph constructed where the x-axis represented the log<sub>10</sub> value of the corresponding reciprocal dilution and the y-axis represented the percentage survival. The results of this assay are shown in Table 7.

25

TABLE 5:

## Immunogenicity of Pneumococcal 14-Tetanus Toxoid Conjugates

Vaccine/ adjuvant	Elisa day0	Elisa day28	Elisa day38	Elisa day59	OP day59
Control / Saline	<50	2,250	29,000	32,600	3,100
Control / Alum	<50	18,200	99,000	265,000	25,000
Pn14-TT/ Saline	<50	4,600	89,000	59,200	3,900
Pn14-TT/ Alum	<50	27,000	185,000	251,000	26,000
PBS/ Alum	<50	<50	<50	<50	<50

Control vaccine was a type 14 polysaccharide-tetanus toxoid conjugate prepared by reductive amination. Pn14-TT conjugate was the product of direct coupling between an N-Acryloylated type 14 pneumococcal polysaccharide and tetanus toxoid.

For this study groups of 10 CD1 mice (Charles River Laboratory) aged 6-8 weeks, were injected subcutaneously with 2.0 µg of conjugated polysaccharide on days 0, 28 and 38. The animals were bled on days 0, 28, 38 and exsanguinated on day 59. ELISAs were performed using Pn14 polysaccharide-HSA conjugate prepared by reductive amination. The ELISA titers reported in Table 5 represent total IgGs. The reported OP titers are against pneumococcal type 14 strain.

**TABLE 6:**  
**Immunogenicity of GBS Type III Conjugates**

Vaccine/ Adjuvant	Elisa day 0	Elisa day 21	Elisa day 42	Elisa day 52	OP day 52
Control conjugate/ Alum	<50	900	1,800	3,000	170
GBS III-TT / Alum	<50	500	8,500	25,000	3,100
PBS / Alum	<20	<20	<20	<20	<20

15

Control conjugate vaccine was a GBS type III-Tetanus toxoid conjugate prepared by reductive amination of a periodate oxidized GBS type III polysaccharide and tetanus toxoid. GBS III-TT conjugate was the product of direct coupling between an N-Acryloylated type III polysaccharide and tetanus toxoid.

20

For this study groups of 10 CD1 mice (Charles River Laboratory) aged 6-8 weeks, were injected subcutaneously with 2 µg of conjugated polysaccharide on days 0, 21, and 42. Mice were bled on days 0, 21, 42, and exsanguinated on day 52. ELISA titers were measured using a GBS type III polysaccharide coupled to human serum albumin, titers given in Table 6 represent total IgGs to the type III polysaccharide.

25

**TABLE 7:**  
**Immunogenicity of E. Coli K1 Conjugates**

Vaccine / Adjuvant	Elisa day 0	Elisa day 28	Elisa day 42	Elisa day 52	SBA day 52
K1-rPorB II / Alum Control Lot1	<50	1,000	39,000	106,000	450
K1-rPorB II / Alum Control Lot 2	<50	450	124,000	250,000	1,000
K1-rPorB I / Alum Lot 1	<50	220	27,000	96,000	810
K1-rPorB I / Alum Lot 2	ND	ND	24,000	71,000	3,800
K1-S-rPorB / Alum Lot 1	ND	ND	45,000	114,000	2,600
K1-S-rPorB / Alum Lot2	ND	ND	41,000	94,000	1,700
PBS / Alum	<50	<50	<50	<50	<50

5 Control vaccine (K1-rPorB II) was the product of reductive amination between a periodate-oxidized N-Acryloylated K1 polysaccharide and tetanus toxoid. K1-rPorB I vaccine was the product of direct coupling of an N-Acryloylated K1 polysaccharide and tetanus toxoid. K1-S-rPorB was the product of direct coupling of the thiolated porin rPorB and the N-Acryloylated K1 polysaccharide.

10 For these studies groups of 10 CD1 mice (4-6 weeks old) from Charles River laboratory were immunized intraperitoneally on days 0, 28, and 42. Mice were bled on days 0, 28, 42, and then exsanguinated on day 52. ELISAs titers were measured using an N-Propionylated K1 polysaccharide coupled to human serum albumin. Titers shown in Table 7 represent total IgGs to the modified N-Propionylated K1 polysaccharide. Serum bactericidal activities (SBA) against *N.meningitidis* group B serotype 15 H44/76 strain, for the day 52 bleed, are also shown in Table 7.

**TABLE 8:**  
**Immunogenicity of GCMP Conjugates**

Vaccine/ Adjuvant	ELISA day 38	SBA day 38
GCMP-S-rPorB/ Alum Lot1	44,000	2,100
GCMP-S-rPorB/ Alum Lot2	43,000	2,800
PBS/ Alum	<50	<50

- GCMP-S-rPorB was the product of direct coupling between the N-Acryloylated group C meningococcal polysaccharide (GCMP) and the thiolated rPorB.
- 5 For these animal studies groups of 10 outbred Swiss Webster female mice (6-8 weeks old) from HSD were injected s.c. with 2 µg of conjugated polysaccharide per dose on days 0 and 28. Animals were exsanguinated on day 38. ELISA titers to the group C polysaccharide are measured using a GCMP coupled to human serum albumin. Serum
- 10 bactericidal titers are obtained using the meningococcal C11 reference strain.

## References

1. Anderson, P., G. Peter, R.B. Johnson, L.H. Wetterlow and D.H. Smith. 1972. Immunization of humans with polyribosylphosphate, the capsular antigen of *Haemophilus influenzae* type b. *J.Clin.Invest.* 51:39-44.
- 5 2. Avery, O.T. and W.F. Goebel. 1931. Chemo-immunological studies on conjugated carbohydrate-proteins V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of type 3 pneumococcus with foreign protein. *J.Exp.Med.* 54:437-447.
3. Baker, C.J. and D.L. Kasper. 1985. Group B streptococcal vaccines.  
10 *Rev.Inf.Dis.* 7:458-467.
4. Baker, C.J., M.A. Rench, M.S. Edwards, R.J. Carpenter, B.M. Hays and D.L. Kasper. 1988. Immunization of pregnant women with a polysaccharide vaccine of group B *Streptococcus*. *N.Engl.J.Med.* 319:1180-1185.
5. Baker, C.J., M.A. Rench and D.L. Kasper. 1990. Response to Type III  
15 polysaccharide in women whose infants have had invasive Group B streptococcal infection. *New Engl.J.Med.* 322:1857-1860.
6. Baltimore, R.S., D.L. Kasper and J. Vecchitto. 1979. Mouse protection test for group B *Streptococcus* type III. *J.Infect.Dis.* 140:81-86.
7. Bednar, B. and J.P. Hennessey. 1993. Molecular size analysis of  
20 capsular polysaccharide preparations from *Streptococcus pneumoniae*. *Carbohydr.Res.* 243:115-130.
8. Beri, R.G., J. Walker, E.T. Reese and J.E. Rollings. 1993. Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique. *Carbohydr.Res.* 238:11-26.
- 25 9. Bradford, M.M.. 1976. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt.Biochem.* 72:248-254.
10. D'Ambra, A.J., J.E. Baugher, P.E. Concannon, R.A. Pon and F. Michon. 1997. Direct and indirect methods for molar-mass analysis of fragments of  
30 the capsular polysaccharide of *Haemophilus influenzae* type b. *Anal.Biochem.* 250(2):228-236.



11. Dick, W.E., Jr. and M. Beurret. 1989. Glycoconjugates of bacterial carbohydrate antigens. p. 48-114. In: Conjugate Vaccines Contributions to Microbiology and Immunology. J.M. Cruse and R.E. Lewis, Jr., (eds) S.Karger, Basel.
12. Dillon, H.C., Jr., S. Khare and B.M. Gray. 1987. Group B streptococcal carriage and disease: A 6-year prospective study. *J.Pediat.* 110:31-36.
13. Goebel, W.F. and O.T. Avery. 1931. Chemo-immunological studies on conjugated carbohydrate-proteins IV. The synthesis of the p-aminobenzyl ether of the soluble specific substance of type 3 pneumococcus and its coupling with protein. *J.Exp.Med.* 54:431-436.
14. Gold, R., M.L. Lepow, I. Goldschneider, T.L. Draper and E.C. Gotschlich. 1975. Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants. *J.Clin.Invest.* 56:1536-1547.
15. Gold, R., M.L. Lepow, I. Goldschneider and E.C. Gotschlich. 1977. Immune response of human infants to polysaccharide vaccines of Groups A and C *Neisseria meningitides*. *J.Infect.Dis.* 136S:S31-S35.
16. Gold, R.M., M.L. Lepow, I. Goldschneider, T.F. Draper and E.C. Gotschlich. 1978. Antibody responses of human infants to three doses of group A *Neisseria meningitides* vaccine administered at two, four and six months of age. *J.Infect.Dis.* 138:731-735.
17. Hennessey, J.P., B. Bednar and V. Manam. 1993. Molecular size analysis of *Haemophilus influenzae* type b capsular polysaccharide. *J.Liq.Chromat.* 16:1715-1729.
18. Howard, J.G., G.H. Christie, B.M. Courtenay, E. Leuchars and A.J.S. Davies. 1971. Studies on immunological paralysis. VI. Thymic-independence of tolerance and immunity to type III pneumococcal polysaccharide. *Cell.Immunol.* 2:614-626.
19. Jennings, H.J., E. Katzenellenbogen, C. Lugowski and D.L. Kasper. 1983. Structure of the native polysaccharide antigens of type Ia and type Ib Group B *Streptococcus*. *Biochemistry* 22:1258-1263.
20. Jennings, H.J., K.-G. Rosell and D.L. Kasper. 1980. Structural determination and serology of the native polysaccharide antigen of type III group B *Streptococcus*. *Can.J.Biochem.* 58:112-120.

21. Jennings, H.J., K.-G. Rosell and D.L. Kasper. 1980. Structure and serology of the native polysaccharide antigen of type Ia group B Streptococcus. *Proc.Nat.Acad.Sci.USA*. 77:2931-2935.
22. Jennings, H.J., K.-G. Rosell, E. Katzenellenbogen and D.L. Kasper.  
5 1983. Structural determination of the capsular polysaccharide antigen of type II Group B Streptococcus. *J.Biol.Chem.* 258:1793-1798.
23. Jennings, H.J. and R.K. Sood. 1994. Synthetic glycoconjugates as human vaccines. p. 325-371. In: Y.C. Lee and R.T. Lee, *Neoglycoconjugates: Preparation and applications*. Academic Press, New York.
- 10 24. Kasper, D.L., C.J. Baker, R.S. Baltimore, J.H. Crabb, G. Schiffman and H.J. Jennings. 1979. Immunodeterminant specificity of human immunity to type III group B Streptococcus. *J.Exp.Med.* 149:327-339.
25. Knobloch, J.E. and P.N. Shaklee. 1997. Absolute molecular weight of low-molecular-weight heparins by size-exclusion chromatography with multiangle  
15 laser light scattering detection. *Anal.Biochem.* 245:231-241.
26. Lancefield, R.C.. 1933. A serological differentiation of human and other groups of haemolytic streptococci. *J.Exp.Med.* 57:571-595.
27. Lancefield, R.C.. 1938. A micro-precipitin technique for classifying hemolytic streptococci and improved methods for producing antigen.  
20 *Proc.Soc.Exp.Biol.and Med.* 38:473-478.
28. Lancefield, R.C., M. McCarty and W.N. Everly. 1975. Multiple mouse-protective antibodies directed against group B streptococci: Special reference to antibodies effective against protein antigens. *J.Exp.Med.* 142:165-179.
29. Madoff, L.C., L.C. Paoletti, J.Y. Tai and D.L. Kasper. 1994. Maternal  
25 immunization of mice with Group B streptococcal type III polysaccharide-beta C protein conjugate elicits protective antibody to multiple serotypes. *J.Clin.Invest.* 94:286-292.
30. Marques, M.B., D.L. Kasper, A. Shroff, F. Michon, H.J. Jennings and M.R. Wessels. 1994. Functional activity of antibodies to the group B polysaccharide  
30 of group B streptococci elicited by a polysaccharide-protein conjugate vaccine. *Infect.Immun.* 62:1593-1599.

31. Mäkelä, P.R.H., H. Peltola, H. Kayhty, et al. 1977. Polysaccharide vaccines of group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: A field trial in Finland. *J.Infect.Dis.* 136:S43-50.
32. Michon, F., J.R. Brisson, A. Dell, D.L. Kasper and H.J. Jennings. 1988. Multiantennary group-specific polysaccharide of group B *Streptococcus*. *Biochem.* 27:5341-5351.
33. Peltola, A., H. Käyhty, A. Sivonen and P.R.H. Mäkelä. 1977. *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: A double blind field study of 100,000 vaccines 3 months to 5 years of age in Finland. *Pediatrics* 60:730-737.
34. Peltola, H., P.R.H. Mäkelä, H. Jousimies, et al. 1977. Clinical efficacy of meningococcal group A vaccine in children three months to five years of age. *N.Engl.J.Med.* 297:686-691.
35. Reuter, G. and R. Schauer. 1994. Determination of sialic acids. p. 168-199. In: W.J. Lennarz and G.W. Hart, *Methods in Enzymology* Vol. 230 *Techniques in Glycobiology*. Academic Press, New York.
36. Robbins, J.B. and R. Schneerson. 1990. Polysaccharide-protein conjugates: A new generation of vaccines. *J.Infect.Dis.* 161:821-832.
37. Smith, A.L. and J. Haas. 1991. Neonatal Bacterial Meningitis. p. 313-333. In: W.M. Scheld, R.J. Whitley and D.T. Durack, *Infections of the Central Nervous System*. Raven Press, Ltd., New York.
38. Tsunashima, T., K. Moro, B. Chu and T.-Y. Liu. 1978. Characterization of group C meningococcal polysaccharide by light-scattering spectroscopy. III. Determination of molecular weight, radius of gyration, and translational diffusional coefficient.. *Biopolymers* 17:251-265.
39. von Hunolstein, C., L. Nicolini, S. D'Ascenzi, C. Volpe, G. Alfarone and G. Orefici. 1993. Sialic acid and biomass production by *Streptococcus agalactiae* under different growth conditions. *Appl.Microbiol.Biotechnol.* 38:458-462.
40. Wessels, M.R., W.J. Benedi, H.J. Jennings, F. Michon, J.L. DiFabio and D.L. Kasper. 1989. Isolation and characterization of type IV group B *Streptococcus* capsular polysaccharide. *Infect.Immun.* 57:1089-1094.

41. Wessels, M.R., J.L. DiFabio, V.J. Benedi, et al. 1991. Structural determination and immunochemical characterization of the type V group B Streptococcus capsular polysaccharide. *J.Biol.Chem.* 266:6714-6719.
42. Wessels, M.R., L.C. Paoletti, D.L. Kasper, et al. 1990.
- 5 Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III group B Streptococcus. *J.Clin.Invest.* 86:1428-1433.
43. Wessels, M.R., L.C. Paoletti, A.K. Rodewald, et al. 1993. Stimulation of protective antibodies against type Ia and Ib group B streptococci by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. *Infect.Immun.* 61:4760-4766.
- 10 44. Wessels, M.R., V. Pozsgay, D.L. Kasper and H.J. Jennings. 1987. Structure and immunochemistry of an oligosaccharide repeating unit of the capsule polysaccharide of Type III Group B Streptococcus: A revised structure for the Type III Group B streptococcal polysaccharide antigen. *J.Biol.Chem.* 262:8262-8267.
45. Wyle, S.A., M.S. Artenstein, B.L. Brandt, et al. 1972. Immunologic  
15 response of man to group B meningococcal polysaccharide vaccines. *J.Infect.Dis.* 126:514-522.
46. Romanowska, A., S.J. Meunier, F.D. Tropper, C.A. LaFerriere, and R. Roy. 1994. Michael Additions for Synthesis of Neoglycoproteins. *Methods in Enzymology.* Vol. 242:90-101.
- 20 47. Orellana, A., and C.B. Hirshberg. 1994. Molecular Cloning and Expression of a Glycosaminoglycan N-Acetylglucosaminyl N-Deacetylase/N-Sulfotransferase from a Heparin-producing Cell Line. *J. Biol. Chem.* Vol 269, No. 3 pp 2270-2276.
48. Cheung, W-F., I.Eriksson, M.Kusche-Gullberg, U. Lindahl, and L.  
25 Kjéllén, 1996. Expression of the Mouse Mastocytoma Glucosaminyl N-Deacetylase/N-sulfotransferase in Human Kidney 293 Cells Results in Increased N-Sulfation of Heparan Sulfate. *Biochem* 35: 5250-5256.
49. Kusche-Gullberg, M., I. Eriksson, D. Sandback Pikas, and L. Kjellen  
1998. Identification and Expression in Mouse of Two Heparan Sulfate Glucosaminyl  
30 N-Deacetylase/N-Sulfotransferase Genes. *J. Biol. Chem.* Vol 273, No. 19: 11902-11907.

50. Finke, A., D. Bronner, A. V. Nikolaev, B. Jann, and K. Jann. 1991. Biosynthesis of the *Escherichia coli* K5 Polysaccharide, a Representative of Group II Capsular Polysaccharides: Polymerization In Vitro and Characterization of the Product. J. Bacteriology 173, No. 13: 4088-4094.
- 5 51. Dubois, M. et al 1965. Colormetric Method for the Determination of Sugars and Related Substance. Analytic. Chem. Vol. 28: 350-366.

We claim:

1. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising an N-propionated polysaccharide or N-propionated oligosaccharide directly conjugated to a protein at the  $\beta$ -position of the propionate moiety.
2. A polysaccharide-protein conjugate according to claim 1 wherein the protein comprises at least one lysine or cysteine residue.
3. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from bacteria, yeast, cancer cells, or chemically synthesized.
4. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.
5. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from Group B streptococcus selected from the group consisting of serotype Ia, serotype Ib, serotype II, serotype III, serotype V, serotype VIII, and combinations thereof.
6. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 4 wherein the polysaccharide or oligosaccharide is derived from a Meningococcus group selected from the group consisting of group B, group C, group Y, group W135, and combinations thereof.
7. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 4 wherein the polysaccharide or oligosaccharide is derived from *E. coli* K1, *E. coli* K92, Pneumococcus type 4, Pneumococcus type 14, Streptococcus group A, Streptococcus group C, or combinations thereof.
8. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a *Neisseria meningitidis* outer membrane protein, pneumolysoid, C- $\beta$  protein from group B *Streptococcus* and non-IgA-binding C- $\beta$  protein from group B *Streptococcus*.

9. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 8 wherein the protein is recombinantly produced.

10. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 9 wherein the protein is recombinant *N.*

5. *meningitidis* outer membrane protein.

11. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide comprises a glycosaminoglycan.

10. 12. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide comprises glycosyl residues of a structural repeating unit having at least one free amino group or N-acyl group.

15. 13. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 12 wherein the glycosyl residue is selected from the group consisting of glucosamine, galactosamine, mannosamine, fucosamine and sialic acid.

20. 14. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the N-propionated polysaccharide or N-propionated oligosaccharide is directly conjugated to an  $\epsilon$ -free amino group of a lysine residue or a thiol group of a cysteine residue of the protein.

25. 15. A polysaccharide-protein conjugate comprising N-propionated *Streptococcus pneumoniae* type 14 polysaccharide-tetanus toxoid conjugate, N-propionated Group B streptococcus type III polysaccharide-tetanus toxoid conjugate, N-propionated Group B Streptococcus type II polysaccharide-tetanus toxoid conjugate, N-propionated *E. coli* K1 polysaccharide-protein conjugate, or N-propionated meningococcal C polysaccharide-tetanus toxoid conjugate.

16. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate produced by a method comprising:

30. A) de-N-acetylating an isolated polysaccharide or oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide with an acryloylating reagent to form an N-propionated polysaccharide or an N-propionated oligosaccharide, and

5 C) directly conjugating the N-propionated polysaccharide or an N-propionated oligosaccharide to a protein to form the polysaccharide-protein conjugate or the oligosaccharide protein conjugate.

17. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 16 wherein the polysaccharide or oligosaccharide is derived from bacteria, yeast, cancer cells or chemical synthesis.

10 18. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted at a pH of about 7.0.

19. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted at a pH above 9.

15 20. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted in a reagent selected from the group consisting of phosphate, carbonate/bicarbonate buffer and borate buffer.

20 21. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the de-N-acetylating reagent is a base or an enzyme and the acryloylating reagent is selected from the group consisting of N-acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

25 22. A pharmaceutical composition comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claims 1 or 16 and a pharmaceutically acceptable carrier.

23. The pharmaceutical composition according to claim 22 further comprising an adjuvant.

24. The pharmaceutical composition according to claim 23 wherein the adjuvant is selected from the group consisting of alum or stearyl tyrosine.

30 25. The pharmaceutical composition according to claim 22 further comprising a second component, said second component selected from the group consisting of DTP, DTaP, Td, DTaP-Hib, DTaP-IPV-Hib, and combinations thereof.



26. A immunogen comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claims 1 or 16, said immunogen elicits a polysaccharide-specific or an oligosaccharide-specific immune response.

27. The immunogen according to claim 26, wherein the immune response is generation of polysaccharide-specific or an oligosaccharide-specific immunoglobulin.

28. The immunogen according to claim 27 wherein the immunoglobulin is IgG, IgM, IgA or combinations thereof.

29. A method of making a  $\beta$ -propionamido-linked polysaccharide-protein conjugate or a  $\beta$ -propionamido-linked oligosaccharide-protein conjugate comprising:

A) de-N-acetylating a polysaccharide or an oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or de-N-acetylated oligosaccharide with an acryloylating reagent to form a  $\beta$ -propionated polysaccharide or a  $\beta$ -propionated oligosaccharide, and

C) directly conjugating the  $\beta$ -propionated polysaccharide or the  $\beta$ -propionamido oligosaccharide to a protein to form the  $\beta$ -propionamido-linked polysaccharide-protein or  $\beta$ -propionamido-linked oligosaccharide-protein conjugate.

30. The method of claim 29, wherein the de-N-acetylating reagent is a base or enzyme.

31. The method of claim 29 wherein the de-N-acetylating reagent is selected from the group consisting of NaOH, KOH and KiOH.

32. The method of claim 29, wherein the acryloylating reagent is selected from the group consisting of acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

33. The method of claim 29, wherein the polysaccharide or oligosaccharide is derived from bacteria, yeast, cancer cells or chemical synthesis.

34. The method of claim 29 wherein the polysaccharide or oligosaccharide is derived from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

35. The method of claim 29 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a neisserial outer membrane protein, pneumolysoid, and C- $\beta$  protein from group B Streptococcus and non-IgA binding C- $\beta$  protein from group B Streptococcus.

36. The method of Claim 35, wherein the protein is recombinantly produced.

37. A vaccine comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 or 16, wherein said vaccine provides protective immunity against a disease causing organism or cell.

38. A vaccine according to claim 37 wherein the disease causing organism or cell is selected from the group consisting of bacteria, yeast, and cancer cell.

39. A vaccine according to claim 38 wherein the bacteria is *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

40. A vaccine according to claim 37 further comprising a second immunogen in combination with the polysaccharide-protein conjugate or oligosaccharide-protein conjugate said second immunogen selected from the group consisting of DTP, DTaP, Td, DTaP, Hib, DTaP-IPV-Hib and combinations thereof.

41. A method of immunizing a mammal against a disease causing organism or disease causing cell comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

42. A method of immunizing a mammal against *Streptococcus pneumoniae* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

43. A method of immunizing a mammal against Group B Streptococcus comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

44. A method of immunizing a mammal against Group B *Neisseria meningitidis* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

45. A method of immunizing a mammal against Group C *Neisseria meningitidis* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

46. A method of immunizing a mammal against *Haemophilus influenzae* type B comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

47. A method of eliciting an antibody response to a polysaccharide or an oligosaccharide in a mammal comprising administering of an effective amount of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 1 or 16.

48. An immunoglobulin or antigen-binding fragment thereof produced according to the method of claim 47.

49. The immunoglobulin according to claim 48, selected from the group consisting of IgG antibody, IgM antibody, IgA antibody and combinations thereof.

50. The immunoglobulin according to claim 49, wherein the antibody is an isolated IgG.

51. An isolated antibody or antigen binding fragment thereof elicited in response to the  $\beta$ -propionamido-linked polysaccharide-protein conjugate or  $\beta$ -propionamido-linked oligosaccharide-protein conjugate according to claim 1 and 16, said antibody or antigen fragment thereof specifically immunoreactive with N-propionated polysaccharide or N-propionated oligosaccharide and immunoreactive with a native N-acetylated polysaccharide from which the  $\beta$ -propionated polysaccharide or  $\beta$ -propionated oligosaccharide was derived.

52. The antibody or antigen binding fragment thereof according to claim 51 wherein the native N-acetylated polysaccharide is a component of bacteria, yeast or cancer cells.

53. The antibody or antigen binding fragment thereof according to claim 52 wherein the polysaccharide is derived from *Escherichia coli*.

Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

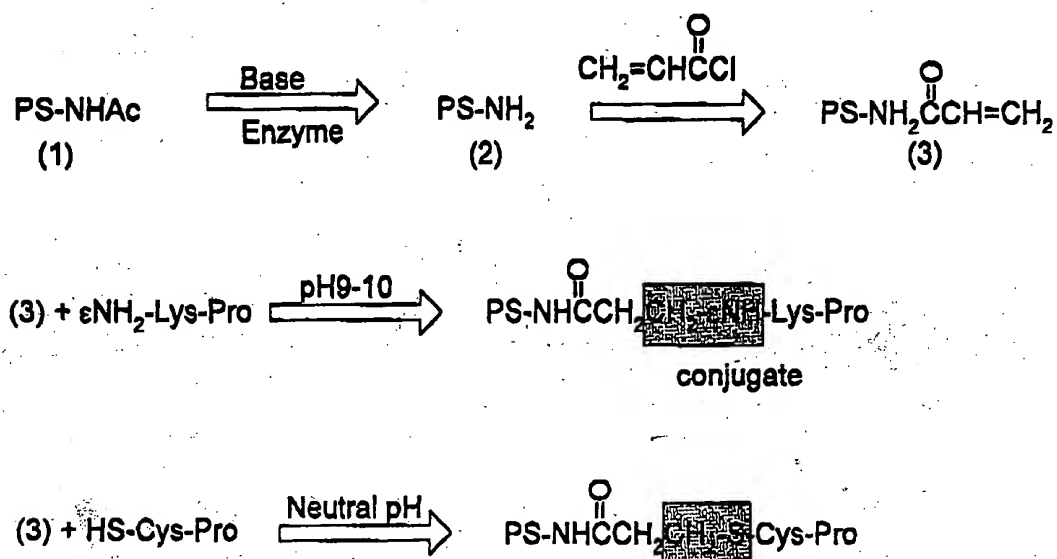
54. The antibody or antigen binding fragment thereof according to claim 51 wherein the antibody is recombinantly produced.

5 55. A method of passive immunization against a disease causing organism or disease causing cells comprising administration of an effective amount of the immunoglobulin or antibody according to claim 48 or 51, said amount is sufficient to inhibit or kill the disease causing organism or disease causing cells.

10 56. The method of passive immunization according to claim 55 wherein the immunoglobulin is an isolated IgG antibody or antigen binding fragment thereof.

57. The method of passive immunization according to claim 55 wherein the immunoglobulin is an isolated IgM antibody or antigen binding fragment thereof.

15 58. The method of passive immunization according to claim 55 wherein the immunoglobulin is an isolated IgA antibody or antigen binding fragment thereof.

**FIGURE 1**

PS = Polysaccharide

Pro = Protein

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/18982

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/385 A61K39/095 A61K39/108 A61K39/09 C08B37/00  
C08L5/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C08B C08L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40239 A (CANADA NAT RES COUNCIL) 19 December 1996 (1996-12-19) page 1, line 24 -page 9, line 35; examples 1-8; tables 1-3	1-58
X	US 5 576 002 A (JENNINGS HAROLD J ET AL) 19 November 1996 (1996-11-19)	15, 48-58
Y	column 3, line 7 -column 11, line 5; claims 1-38	1-14, 16-47
X	US 4 727 136 A (JENNINGS HAROLD J ET AL) 23 February 1988 (1988-02-23)	15, 48-58
Y	column 1, line 1 -column 8, line 39	1-14, 16-47
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

25 February 2000

Date of mailing of the international search report

17.03.00

Name and mailing address of the ISA

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Muller-Thomalla, K

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/18982

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JENNINGS H J ET AL: "Induction of meningococcal group B polysaccharide-specific IgG antibodies in mice by using an N-propionylated B polysaccharide-tetanus toxoid conjugate vaccine." JOURNAL OF IMMUNOLOGY, (1986 SEP 1) 137 (5) 1708-13. , XP002052677	15,48-58
Y	abstract, "materials and methods" starting at page 1708 and "discussion" starting at page 1711	1-14, 16-47
X	JENNINGS H J ET AL: "Unique intermolecular bactericidal epitope involving the homosialopolysaccharide capsule on the cell surface of group B Neisseria meningitidis and Escherichia coli K1." JOURNAL OF IMMUNOLOGY, (1989 MAY 15) 142 (10) 3585-91. , XP000872571	15,48-58
Y	abstract	1-14, 16-47
X	FUSCO P C ET AL: "Preclinical evaluation of a novel group B meningococcal conjugate vaccine that elicits bactericidal activity in both mice and nonhuman primates." JOURNAL OF INFECTIOUS DISEASES, (1997 FEB) 175 (2) 364-72. , XP000291371	15,48-58
Y	abstract page 364, column 2, paragraph 3 -page 366, column 2, paragraph 2	1-14, 16-47
X	FUSCO, PETER C. ET AL: "Meningococcal vaccine development: a novel approach" EXPERT OPIN. INVEST. DRUGS (1998), 7(2), 245-252 , XP000872557	15,48-58
Y	abstract; figure 1 page 245, line 1 -page 247, column 1, paragraph 1	1-14, 16-47
X	GRANOFF D M ET AL: "Bactericidal monoclonal antibodies that define unique meningococcal B polysaccharide epitopes that do not cross-react with human polysialic acid." JOURNAL OF IMMUNOLOGY, (1998 MAY 15) 160 (10) 5028-36. , XP000872887	15,48-58
Y	abstract and "materials and methods" starting at page 5029	1-14, 16-47
-/-		

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/18982

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JENNINGS H J ET AL: "Chemically modified Group B meningococcal polysaccharides as human vaccines" PROCEEDINGS OF THE NATIONAL MEETING: SYMPOSIUM ON THE APPLICATIONS AND MODIFICATIONS OF INDUSTRIAL POLYSACCHARIDES, NL, AMSTERDAM, ELSEVIER, vol. MEETING 193, 1987, pages 149-156, XP002016901	15, 48-58
Y	page 150, line 9 -page 156, line 3	1-14, 16-47
A	US 4 356 170 A (JENNINGS HAROLD J ET AL) 26 October 1982 (1982-10-26) claims 1-26; examples 1-3	1-58



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/18982

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 41-47 and 55-58 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Application No

PCT/US 99/18982

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640239 A	19-12-1996	US 5811102 A	22-09-1998
		AU 706053 B	10-06-1999
		AU 5993796 A	30-12-1996
		BR 9609229 A	27-07-1999
		CA 2223567 A	19-12-1996
		CZ 9703914 A	13-05-1998
		EP 0831898 A	01-04-1998
		HU 9802664 A	01-02-1999
		JP 11506491 T	08-06-1999
		NO 975547 A	09-02-1998
		PL 323862 A	27-04-1998
		US 5969130 A	19-10-1999
US 5576002 A	19-11-1996	US 5683699 A	04-11-1997
		US 5902586 A	11-05-1999
		AT 121947 T	15-05-1995
		AU 641715 B	30-09-1993
		AU 6898791 A	18-07-1991
		CA 2071811 A	15-06-1991
		WO 9108772 A	27-06-1991
		CN 1055541 A	23-10-1991
		CN 1100428 A, B	22-03-1995
		CN 1100656 A	29-03-1995
		CN 1096516 A, B	21-12-1994
		CZ 9006284 A	17-12-1997
		DE 69019164 D	08-06-1995
		DE 69019164 T	07-09-1995
		DK 504202 T	02-10-1995
		EP 0504202 A	23-09-1992
		ES 2071288 T	16-06-1995
		HR 920872 A	30-06-1997
		HU 64480 A	28-01-1994
		IE 68414 B	12-06-1996
		IL 96676 A	12-09-1996
		IN 171747 A	26-12-1992
		JP 2637845 B	06-08-1997
		KR 158436 B	01-12-1998
		NO 305275 B	03-05-1999
		NO 973311 A	14-08-1992
		NZ 236471 A	28-10-1992
		PL 166035 B	31-03-1995
		PL 166659 B	30-06-1995
		RO 111416 A	31-10-1996
		ZA 9010065 A	26-02-1992
US 4727136 A	23-02-1988	CA 1261320 A	26-09-1989
US 4356170 A	26-10-1982	CA 1181344 A	22-01-1985